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Gastrointestinal Cancer

TERT Promoter Hypermethylation in Gastrointestinal Cancer: A Potential Stool Biomarker

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Disclosures of potential conflicts of interest may be found at the end of this article.

Key Words. Cancer biomarkers • Gastrointestinal cancer • Noninvasive assay • Promoter methylation • Telomerase reverse transcriptase

Abstract

Background. There is a high demand for noninvasive screening tools for gastrointestinal cancer (GIC) detection, and GIC-specific markers are required for such purposes. It is established that induction of the telomerase reverse transcriptase gene (*TERT*) coupled with telomerase activation is essential for cancer development/progression and aberrant *TERT* promoter methylation of specific 5′—C—phosphate—G—3′ (CpGs) has been linked to *TERT* induction in oncogenesis. Here we analyzed *TERT* promoter methylation in fecal samples from GIC patients and healthy adults and determined its value as a stool biomarker for GIC detection.

Materials and Methods. Sixty-nine GIC patients (34 colorectal carcinoma and 35 gastric cancer) and 62 healthy adults were recruited and fecal samples were collected. Paired tumors and adjacent non-cancerous tissues from 34 patients and normal mucosa tissues from 12 healthy individuals were collected. *TERT* promoter methylation density was determined using pyrosequencing.

Results. We identified two GIC-specific methylation sites at −218 (CpG site 1) and −210 (CpG site 2) in the *TERT* promoter in tumor tissues. Methylated *TERT* promoter CpG sites 1 and 2 were also detectable in patient stool, while only background levels were observed in healthy individuals. The overall sensitivity reached 52.2% (95% confidence interval [CI]: 48.3–56.0) for fecal methylated TERT promoter assays at 90% specificity, which was comparable to other known stool methylation markers for GIC detection. The combined assays of fecal *TERT* promoter methylation and occult blood (OB) significantly improved sensitivity and specificity in colorectal cancer (area under curves for methylation alone: 0.798, 95% CI: 0.707–0.889 vs. methylation + OB: 0.920, 95% CI: 0.859–0.981; $p = .028$), but not in gastric cancer.

Conclusion. This proof-of-concept study suggests the feasibility of stool *TERT* promoter methylation analyses as an additional tool in noninvasive GIC screening. ***The Oncologist*** 2017;22:1178–1188

Implications for Practice: Induction of telomerase reverse transcriptase (TERT) expression coupled with telomerase activation is essential for cancer development/progression, while aberrant TERT promoter methylation has been linked to TERT induction in oncogenesis. We identified two cancer-specific methylation sites (CpG1 and 2) in the TERT promoter in tumors from GIC patients. Methylated TERT promoter CpG sites 1 and 2 were detectable in patient stool, while only background levels were observed in healthy individuals. The sensitivity and specificity was comparable to other known stool methylation markers for GIC detection. This proof-of-concept study suggests the feasibility of stool TERT promoter methylation analyses for noninvasive screening of GIC.

Introduction

Gastrointestinal cancer (GIC), including colorectal carcinoma (CRC) and gastric cancer (GC), is the most common malignancy and leading cause of cancer-related death worldwide [1–3]. Because early diagnosis is the key to improving clinical management and patient outcomes, significant efforts have been made to develop tools for screening or early cancer detection

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[1–3]. Although endoscopic examination is useful for the diagnostics of GIC, it is invasive and expensive. Ideally, accurate, noninvasive tests should be established that may be applied for population screening, patient diagnosis, surveillance, and monitoring of treatment response. In this regard, stool-based approaches have been shown to be promising in screening for GIC [1, 3–5]. However, the effectiveness of stool-based screening, especially for GC, remains to be improved by identifying more reliable discriminating biomarkers [1, 3, 4].

Telomerase is an RNA-dependent DNA polymerase responsible for telomere lengthening, and its rate-limiting, catalytic component is telomerase reverse transcriptase (TERT) [6–8]. *TERT* expression or telomerase activity is undetectable in most normal human cells with limited lifespan. By contrast, the induction of *TERT* expression coupled with telomerase activation is a critical step in malignant transformation because telomere stabilization is required for infinite proliferation of tumor cells [6, 7]. Indeed, *TERT* expression is observed in up to 90% of human malignancies, including GIC [6, 7, 9, 10]. The combined absence in normal tissues and prevalence in cancer makes *TERT* or telomerase a novel cancer biomarker. Interestingly, detection of *TERT* mRNA or telomerase activity in urine was previously demonstrated as a useful diagnostic marker for urothelial bladder carcinoma [11, 12]. *TERT*/telomerase analyses have recently been shown as markers for circulating tumor cells [13]. These observations prompted us to test a noninvasive *TERT*-based screening/diagnostic tool for GIC. However, a direct assessment of *TERT* mRNA or telomerase activity in stool is not feasible; therefore, alternative strategies must be established.

The hot spot *TERT* promoter mutations observed in some malignancies rarely occur in GIC. However, it has been well characterized that *TERT* promoter methylation is required for transcriptional activation of the *TERT* gene in malignant transformation [14–17]. Consistently, various types of cancer cells were found to carry a hypermethylated *TERT* promoter, and the inhibition of DNA methylation led to *TERT* promoter demethylation accompanied by down-regulated *TERT* expression [14, 17–20]. Choi et al. observed that site-specific methylation of CpG nucleotides in the *TERT* promoter region controlled the expression of *TERT* during malignant progression of CRC [10]. They further reported that hyper-methylation of three specific CpG sites was closely related with the increase of *TERT* expression [10]. In sharp contrast, the *TERT* promoter is, in general, unmethylated in normal human cells regardless of *TERT* expression [10, 21–23]. Thus, differential methylation statuses of the *TERT* promoter between normal and cancer cells may serve as a potential discriminating biomarker. A number of DNA-methylation-based fecal biomarkers have been evaluated for noninvasive screening of GIC and promising data have been obtained [1]. However, a fecal *TERT* promoter methylation assay has never been tested as a GIC biomarker. In the present study, we sought to determine whether the *TERT* promoter methylation differs between GIC and normal gastrointestinal tissues, and if so, whether the *TERT* promoter methylation analysis in stool is useful for screening of GIC.

Materials and Methods

Subjects and Specimens

A total of 69 patients with GIC and 62 healthy individuals were recruited for the present study. Paired tissue specimens, including both tumors and normal adjacent mucosa (NAT), were obtained from 34 of the patients with GIC (18 GC and 16 CRC) who had all undergone curative surgery at Qilu Hospital of Shandong University between 2012 and 2014. Normal mucosa samples from 12 healthy individuals were collected in the same hospital in 2014. All the samples were frozen at −80°C until subsequent laboratory analyses. All normal and tumorous samples were histopathologically confirmed, and the presence of tumor tissue within malignant specimens was verified.

Feces from all 69 patients with GIC (35 GC and 34 CRC) and 62 healthy individuals were collected before any colonoscopic or surgical intervention. Patients did not adhere to a special diet or medicine prior to the collection of samples. They were instructed to collect an aliquot of feces using a plastic spoon and to store it in a hermetically sealed plastic container (Suzhou, China, http://www.Suzhou-Suyi-Medical-Devices-CoLtd-in-Suzhou-China). Samples were immediately frozen at −80°C. Occult blood (OB) was determined using the colloidal gold labeled method.

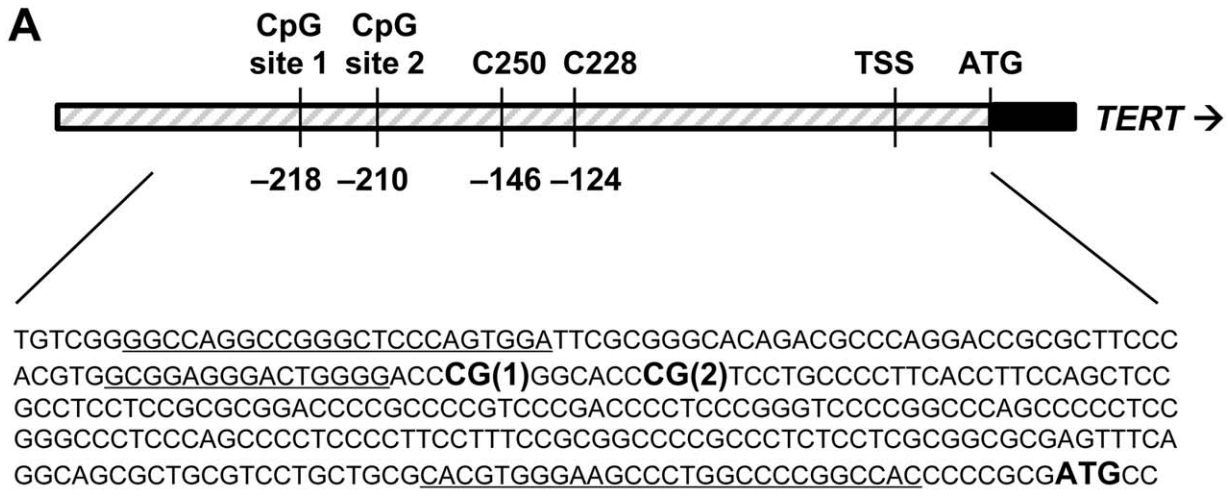
The tissue and stool donation program, and all experimental protocols, were approved by the Ethical Committee of Qilu Hospital of Shandong University. Written informed consent was obtained from all the subjects. The methods were carried out in accordance with the approved guidelines.

DNA Extraction and Bisulfite Treatment

DNA was extracted from frozen tissue samples using a DNeasy blood and tissue kit (Qiagen Ltd, Venlo, The Netherlands, https://www.qiagen.com/us/), and from stool samples using a QIAamp Fast DNA Stool Mini Kit (Qiagen Ltd). DNA concentrations were measured by spectrophotometry and adjusted to 50 ng/mL. Bisulfite modification of genomic DNA was performed with an EpiTect Bisulfite kit (Qiagen Ltd) according to the manufacturer's instruction. Bisulfite conversion of DNA was confirmed in all cases by successful PCR using primers specific to bisulfite-converted DNA.

Pyrosequencing

Polymerase chain reaction (PCR) was performed using the PyroMark PCR Kit (Qiagen Ltd) and the following primers: 5′-TTG GAA GGT GAA GGG GTA G-3′ (forward) and 5′-AAA CCA AAC CCA ACT CCC AAT AAA TT-3′ (reverse biotinylated). Primers were designed using PyroMark Assay Design Software 2.0. The PCR thermal cycling conditions were denaturation at 95°C for 15 minutes, followed by 50 cycles of (a) 94°C for 30 seconds, (b) 58°C for 30 seconds, and (c) 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. Polymerase chain reactions were carried out with 0.5 μl bisulfite-converted genomic DNA for all samples. A biotin-labeled primer (reverse primer) was used to purify PCR products by use of streptavidin-coated Sepharose beads (GE Healthcare, U.K.). Polymerase chain reaction products were bound to Sepharose beads, purified, washed, denatured with a 0.2 M NaOH solution, and washed again. Subsequently, 0.3 μM sequencing primer (5′-GGTGAAGGGGTAGGA-3′) was annealed to the purified single-stranded PCR product, followed by pyrosequencing in a PyroMark Q96 or Q24 (Qiagen Ltd) according to the manufacturer's instructions. To provide an internal control for total bisulfite conversion, a non-CG cytosine in the region for pyrosequencing was included. For each sample, individual methylation density values were determined for CpG site 1 and CpG



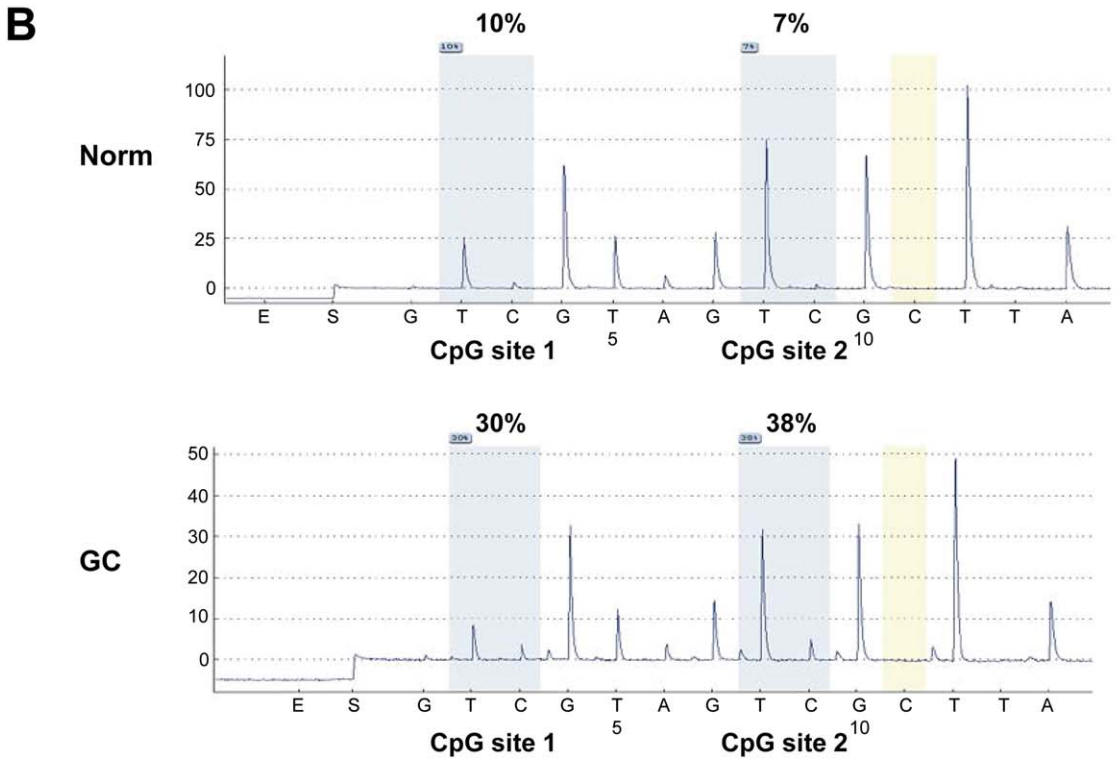


Figure 1. Quantification of methylation density at CpG sites 1 and 2 in the *TERT* promoter region specific to gastrointestinal cancer. **(A)**: Schematic illustration of the *TERT* promoter region with indication of the location for CpG site 1 and 2, hot spot mutations at C228 and C250, TSS, and ATG. The enlarged sequence below shows the precise location of CpG sites 1 and 2 (bolded) in the *TERT* promoter region. Sequences corresponding to PCR primers and sequencing primer are underlined. **(B)**: Representative pyrograms from methylation analyses of normal mucosa from a healthy adult (Norm) and tumor tissue from a patient with GC. The non-GC control is indicated in yellow.

Abbreviations: ATG, start of translation; CpG, 5′—C—phosphate—G—3′; GC, gastric cancer; PCR, polymerase chain reaction; TSS, transcriptional start site.

site 2 (Fig. 1), and, in addition, a methylation index (MetI) was calculated as the mean of CpG site 1 and 2.

Statistical Analysis

The Mann-Whitney U test was used for the unpaired groups to compare methylation levels in fecal DNA from patients and healthy subjects as well as subgroups of cases with different clinical and tumor characteristics. The Wilcoxon rank-sum test was used for the paired groups to calculate two-sided *p* values of *TERT* promoter methylation level in GIC tumors and matched normal mucosa. Receiver operating characteristic (ROC) curves were plotted for potential cut-off values based on the methylation level with respect to the sensitivity for fecal DNA from patients. For the combined methylation/OB data, ROCs were made by binary logistic regression followed by multiple-variables ROS curve assays.

The study was designed to have a power of 90% to first test the hypothesis that the assay would have a sensitivity of 80% or more for CRC (American Joint Committee on Cancer [AJCC] 2003 stages I–IV) and 60% or more for GC (AJCC 2010 stages I–IV); the other hypothesis was to rule out a 5% non-inferiority margin for sensitivity for the detection of CRC and GC with the DNA

Table 1. Patient characteristics and relation to *TERT* promoter methylation

Parameter	Methylation densities in colorectal carcinomas							Methylation densities in gastric cancers						
	No. of cases	CpG[c] site 1		CpG site 2		MetI		No. of cases	CpG site 1		CpG site 2		MetI	
		(%)	*p*	(%)	*p*	(%)	*p*		(%)	*p*	(%)	*p*	(%)	*p*
Age			.268		.557		.344			.727		.831		.77
>62 years	19	12.9		16.6		14.7		17	13.1		18.0		15.5	
≤62 years	15	16.8		18.4		17.6		18	14.0		18.6		16.3	
Gender			.444		.54		.446			.081		.196		.111
Male	19	15.8		18.3		17.0		27	12.4		17.3		14.8	
Female	15	13.1		16.3		14.7		8	17.6		21.8		19.7	
Smoking			.107		.721		.267			.141		.134		.115
Smoking	11	18.7		18.2		18.5		18	11.7		16.2		14.0	
No smoking	23	12.6		17.0		14.8		17	15.5		20.5		18.0	
BMI			.526		.518		.974			.785		.951		.919
≥25 (obesity)	9	12.7		19.1		15.9		14	14.0		18.2		16.1	
<25 (no obesity)	25	15.3		16.8		16.0		21	13.3		18.4		15.8	
OB			.874		.67		.897			.498		.913		.692
With OB	26	14.4		17.8		16.1		6	11.6		18.0		14.8	
Without OB	8	15.1		16.2		15.6		29	14.0		18.4		16.2	
Tumor size[a]			.081		.115		.066							
<7 cm	26	12.9		16.0		14.5		—	—		—		—	
≥7 cm	8	20.1		21.8		21.0		—	—		—		—	
Differentiation			**.041**[b]		.474		.122			.402		**.038**		.116
Intermediate/poor	25	16.7		18.1		17.4			—		—		—	
High	9	8.7		15.5		12.1			—		—		—	
Poor	—	—		—		—		27	14.2		19.9		17.0	
Intermediate/high	—	—		—		—		8	11.6		12.9		12.2	
Stage			**.042**		.675		.169			.129		**.016**		**.042**
Duke's stage A–B	20	11.7		16.8		14.2		—	—	—		—	—	—
Duke's stage C–D	14	18.8		18.2		18.9		—	—	—		—	—	—
Late stage	—	—		—		—		31	14.3		19.8		17.0	
Early stage	—	—		—		—		4	7.8		6.9		7.4	
Metastases										.083		**.038**		**.042**
Metastasis	—	—		—		—		19	15.6		21.0		18.3	
No metastasis	—	—		—		—		16	11.2		15.1		13.1	

[a]Diameter.
[b]Bolded *p* values: with statistically significant difference.
[c]CpG: 5′—C—phosphate—G—3′.
Abbreviations: —, no data; BMI, body mass index; CpG, 5′—C—phosphate—G—3′; MetI, methylation index determined as the mean methylation of site 1 and 2; OB, occult blood; TERT, telomerase reverse transcriptase.

methylation test compared with fecal OB test, at a two-sided type I error rate of .05. Testing of the hypotheses with a power of at least 90% required 30 and 15 CRC and GC cases, respectively.

All *p* values that were reported were calculated in two-sided tests and values less than .05 were considered to be statistically significant.

Results

Demographic and/or Clinical Characteristics of Patients with GIC and Healthy Individuals

Patient information and clinical characteristics are summarized in Table 1. The recruited 69 GIC cases consisted of 35 patients with GC (34 adenocarcinoma, 1 signet ring cell cancer and adenocarcinoma) and 34 patients with CRC (33 adenocarcinoma and 1 neuroendocrine carcinoma). Within the patient group, the mean age was 60.7 years (95% CI: 56.4–65.0) and the mean body mass index (BMI) was 24.9 (95% CI: 23.9–26.1) for patients with GC, whereas patients with CRC had a mean age of 61.8 (95% CI: 57.3–66.3) and a mean BMI of 23.5 (95% CI: 22.3–24.7). The study also included 62 healthy individuals with no evidence of gastrointestinal neoplasia (28 male and 34 female) as normal references, and their mean age and BMI were 60.0 years (95% CI: 56.7–63.4) and 23.9 (95% CI: 22.6–25.2), respectively. There were no significant differences in age and BMI between patients and healthy individuals.

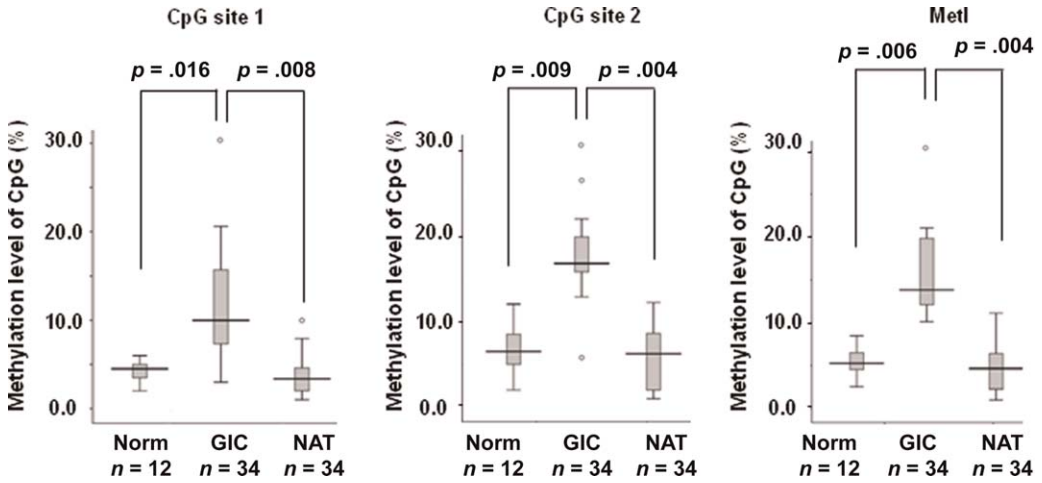


Figure 2. Comparison of *TERT* promoter methylation in GIC tissues and normal mucosa. Box plots showing methylation levels at CpG sites 1 and 2 and the mean of CpG sites 1 and 2 (MetI) in normal mucosa from healthy individuals (Norm), and from matched pairs of GIC tissues and NAT from the same patients. A set of Wilcoxon signed-rank test was used to compare the matched NAT and GIC samples. Mann-Whitney *U* test was applied to compare the GIC patients and healthy individuals.

Abbreviations: CpG, 5′—C—phosphate—G—3′; GIC, gastrointestinal tumor; MET1, methylation index; NAT, normal adjacent mucosa; tissue adjacent to tumor; TERT, telomerase reverse transcriptase.

Table 2. Summary of *TERT* promoter methylation densities in the tissue and fecal sample groups

Samples	No. of cases	CpG site 1 (%) Mean (range)	CpG site 2 (%) Mean (range)	MetI (%) Mean (range)
Tissue samples				
GIC tumor tissue	34	10.5 (8.7–12.3)	17.8 (13.0–22.7)	14.2 (10.9–17.5)
NAT	34	3.8 (2.9–4.7)	5.9 (4.6–7.2)	4.9 (3.8–6.0)
Norm[a]	12	4.0 (3.0–5.0)	6.7 (5.0–8.3)	5.3 (4.3–6.3)
Fecal samples				
CRC	34	14.6 (11.0–18.2)	17.4 (14.2–20.6)	16.0 (12.9–19.1)
GC	35	13.6 (11.0–16.1)	18.3 (15.4–21.2)	15.8 (13.3–18.5)
Norm	62	6.8 (5.4–8.1)	8.9 (6.9–10.9)	7.8 (6.2–9.4)

[a]Healthy individuals.

Abbreviations: CpG, 5′—C—phosphate—G—3′; CRC, colorectal carcinoma; GC, gastric cancer; GIC, gastrointestinal cancer; MetI, methylation index determined as the mean methylation of site 1 and 2; NAT, normal adjacent tissues.

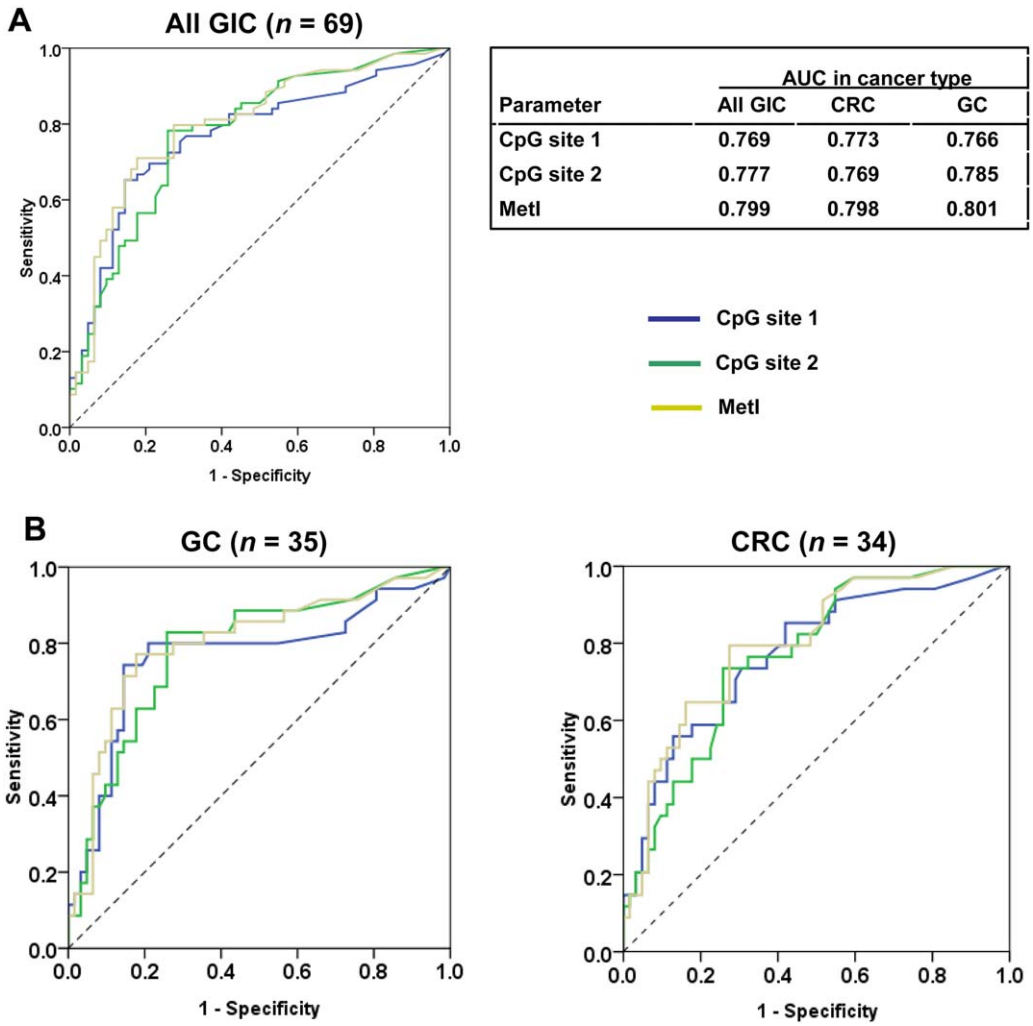
Differential Methylation Statuses of the *TERT* Promoter Between Normal and Malignant Gastrointestinal Tissues

The *TERT* promoter methylation profile in cancer is not identical and different types of cancer may exhibit methylation in different CpGs of the *TERT* promoter. Here we performed pyrosequencing to quantify methylation densities at CpG sites 1 and 2 reported as site-specific in CRC by Choi et al. [10] (Fig. 1A and 1B). The results from analyses of matched specimens of tumor and normal tissue adjacent to tumor (NTAT) obtained from 34 patients with GIC and normal mucosa from healthy controls are illustrated in Figure 2 and summarized in Table 2. These two CpG sites were, in general, unmethylated or methylated at a very low level in NAT and normal mucosa from healthy individuals. In the matched NTAT and GIC tissues, increased methylation was observed in GIC tissues compared with NTAT for CpG site 1, site 2, and MetI (Fig. 2). These results indicate that the methylation status of these two CpGs is capable of distinguishing between normal and malignant gastrointestinal tissues. There was no relationship between the *TERT* promoter methylation level and age using the age of normal controls as a continuous variable ($R^2 = 0.006$ and 0.002, $p = .458$ and .611 for CpG site 1 and 2, respectively).

Detection of *TERT* Promoter Methylation in Fecal DNA from Patients with GIC

We further determined whether these two methylated CpGs could serve as fecal biomarkers for cancer detection. For this purpose, fecal DNA was isolated from patients with GIC and from healthy individuals, bisulphate-treated, and then pyrosequenced. Methylated CpG sites 1 and 2 were indeed detectable in patient stool, while only background levels were found in healthy individuals (Table 2).

Figure 3 shows ROC curves for various cut-offs by *TERT* promoter methylation levels in fecal samples (CpG site 1, site 2, and MetI) from patients with GIC ($n = 69$) and healthy individuals ($n = 62$). For the overall series, the area under curve (AUC) was 0.769 (95% CI: 0.687–0.852) for CpG site 1, 0.777 (95% CI: 0.697–0.877) for site 2, and 0.799 (95% CI: 0.723–0.877) for the MetI. When the specificity was at 90%, the sensitivity reached 0.420 (95% CI: 0.386–0.455) for site 1, 0.391 (95% CI: 0.358–0.425) for site 2 and 0.522 (95% CI: 0.483–0.560) for the MetI (Fig. 3).



Parameter	AUC in cancer type		
	All GIC	CRC	GC
CpG site 1	0.769	0.773	0.766
CpG site 2	0.777	0.769	0.785
MetI	0.799	0.798	0.801

Figure 3. Receiver operating characteristic (ROC) curves of fecal *TERT* promoter methylation in GIC. ROC curves for screening of patients with GIC and healthy adults applying various cut-off levels by *TERT* promoter methylation levels of fecal samples for all GIC, and GC and CRC subgroups. Sensitivity and 1 minus specificity (ROC curves) are shown for CpG sites 1 and 2 and the mean of CpG sites 1 and 2 (MetI). AUC is detailed for each situation at the upper right.

Abbreviations: AUC, area under the curve; CpG, 5′—C—phosphate—G—3′; CRC, colorectal carcinoma; GC, gastric cancer; GIC, gastrointestinal tumor; Met1, methylation index.

We further evaluated the fecal methylated CpG sites 1 and 2 in GC and CRC separately. Overall AUCs in GC were 0.766 (95% CI: 0.655–0.876), 0.785 (95% CI: 0.687–0.883), and 0.801 (95% CI: 0.703–0.899) for site 1, 2, and the MetI, respectively (Fig. 3), whereas those in CRC were 0.773 (95% CI: 0.673–0.872), 0.769 (95% CI: 0.674–0.863), and 0.798 (95% CI: 0.707–0.889) for site 1, 2, and the MetI, respectively (Fig. 3). At 90% specificity, the sensitivity reached 54.3% (95% CI: 49.7–58.8%) and 50.0% (95% CI: 45.6–54.4) for GC and CRC, respectively.

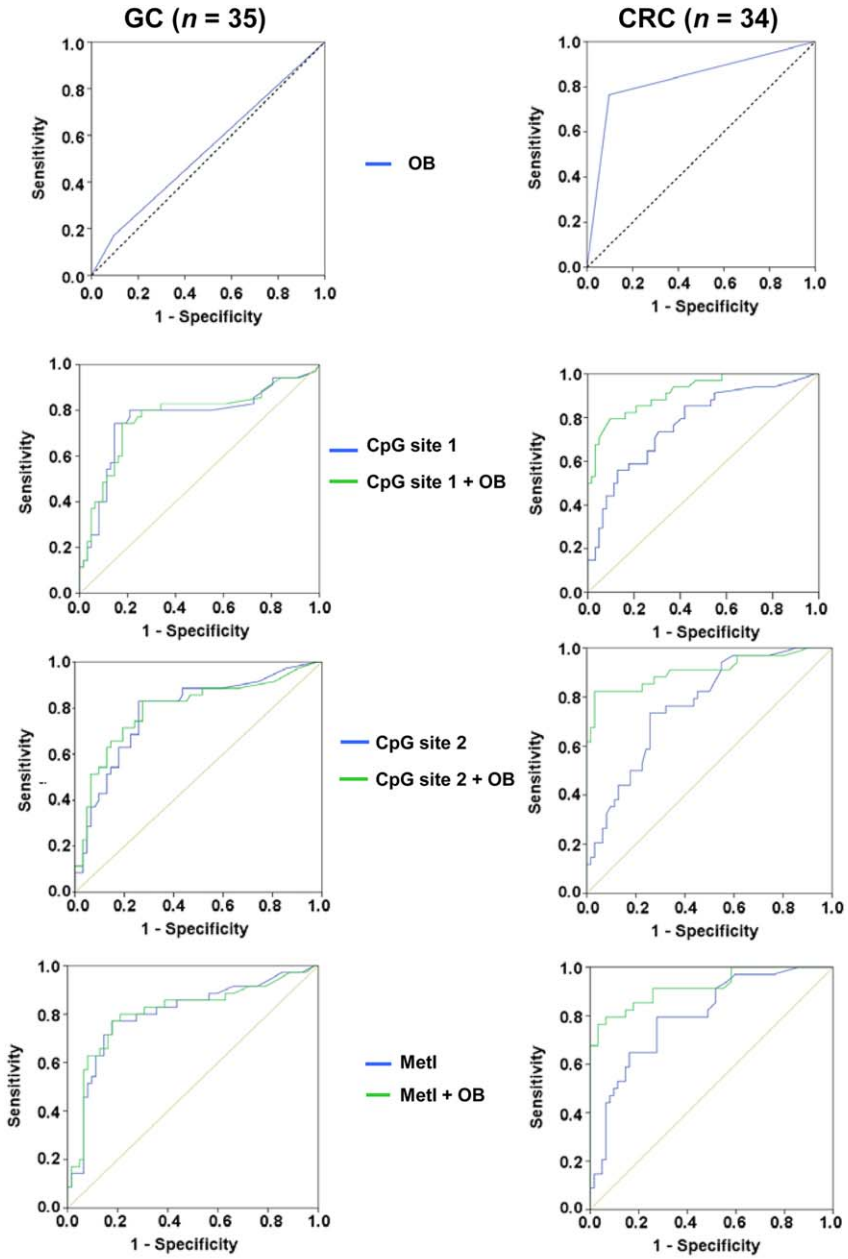
In addition, we observed that the AUC for the combination of sites 1 and 2 was largely comparable to that of the MetI. For CRC, the AUC of sites 1 and 2 combined was 0.794 (95% CI: 0.701–0.887), and 0.798 (95% CI: 0.707–0.889) for the MetI. For GC, the AUC of sites 1 and 2 combined was 0.799 (95% CI: 0.700–0.897), and 0.801 (95% CI: 0.703–0.899) for the MetI.

Combined Assessments of Fecal *TERT* Promoter Methylation and OB in GIC

Fecal OB examination is routinely performed in GIC. Evaluation of the OB results in this cohort of patients showed that AUC for OB in GC was 0.537 (95% CI: 0.416–0.659; Fig. 4), which is lower than that for *TERT* promoter methylation (Fig. 3). In the CRC group, AUC for OB was 0.834 (95% CI: 0.739–0.929; Fig. 4), which is comparable to that for *TERT* methylation (Fig. 3). When the assessment results of *TERT* promoter MetI and OB were combined, AUCs of 0.806 and 0.920 were obtained for GC and CRC, respectively (Fig. 4). Compared with the MetI alone, the detection of both MetI and OB significantly increased AUC in CRC (MetI vs. MetI + OB: 0.798 [95% CI: 0.707–0.889] vs. 0.92 [95% CI: 0.859–0.981], $p = .028$ [Z-test]), but not in GC (0.801 [95% CI: 0.703–0.899] vs. 0.806 [95% CI: 0.707–0.906], $p = .944$).

Association Between Fecal *TERT* Promoter Methylation and Clinical Variables in GIC

We then sought to determine whether the fecal *TERT* promoter methylation status was associated with clinical variables in patients with GIC. In both CRC and GC, the methylation density was independent of age, sex, smoking, and BMI (Table 1). In all 69 GIC, no difference in methylation density was observed in



Parameter	GC AUC (95% CI)	CRC AUC (95% CI)
Occult blood (OB)	0.537 (0.416–0.659)	0.834 (0.739–0.929)
CpG site 1	0.766 (0.655–0.876)	0.773 (0.673–0.875)
CpG site 1 + OB	0.772 (0.665–0.880)	0.917 (0.860–0.974)
CpG site 2	0.785 (0.687–0.883)	0.769 (0.674–0.863)
CpG site 2 + OB	0.796 (0.696–0.896)	0.909 (0.837–0.981)
MetI	0.801 (0.703–0.899)	0.798 (0.707–0.889)
MetI + OB	0.806 (0.707–0.906)	0.920 (0.859–0.981)

Figure 4. Receiver operating characteristic (ROC) curves of OB and in combination with fecal *TERT* promoter methylation. ROC curves are shown for various cut-off levels for fecal samples of patients with GC (left) and CRC (right), respectively. Sensitivity and 1 minus specificity are shown at various threshold values. ROC curves at the top are based on OB analyses alone. Below are shown analyses based on methylation at CpG sites 1, 2, and the mean of sites 1 and 2 (MetI) alone or in combination with OB. AUC and 95% CIs are detailed for each situation at the bottom.

Abbreviations: AUC, area under the curve; CI, confidence interval; CRC, colorectal carcinoma; GC, gastric cancer; Met1, methylation index; OB, occult blood.

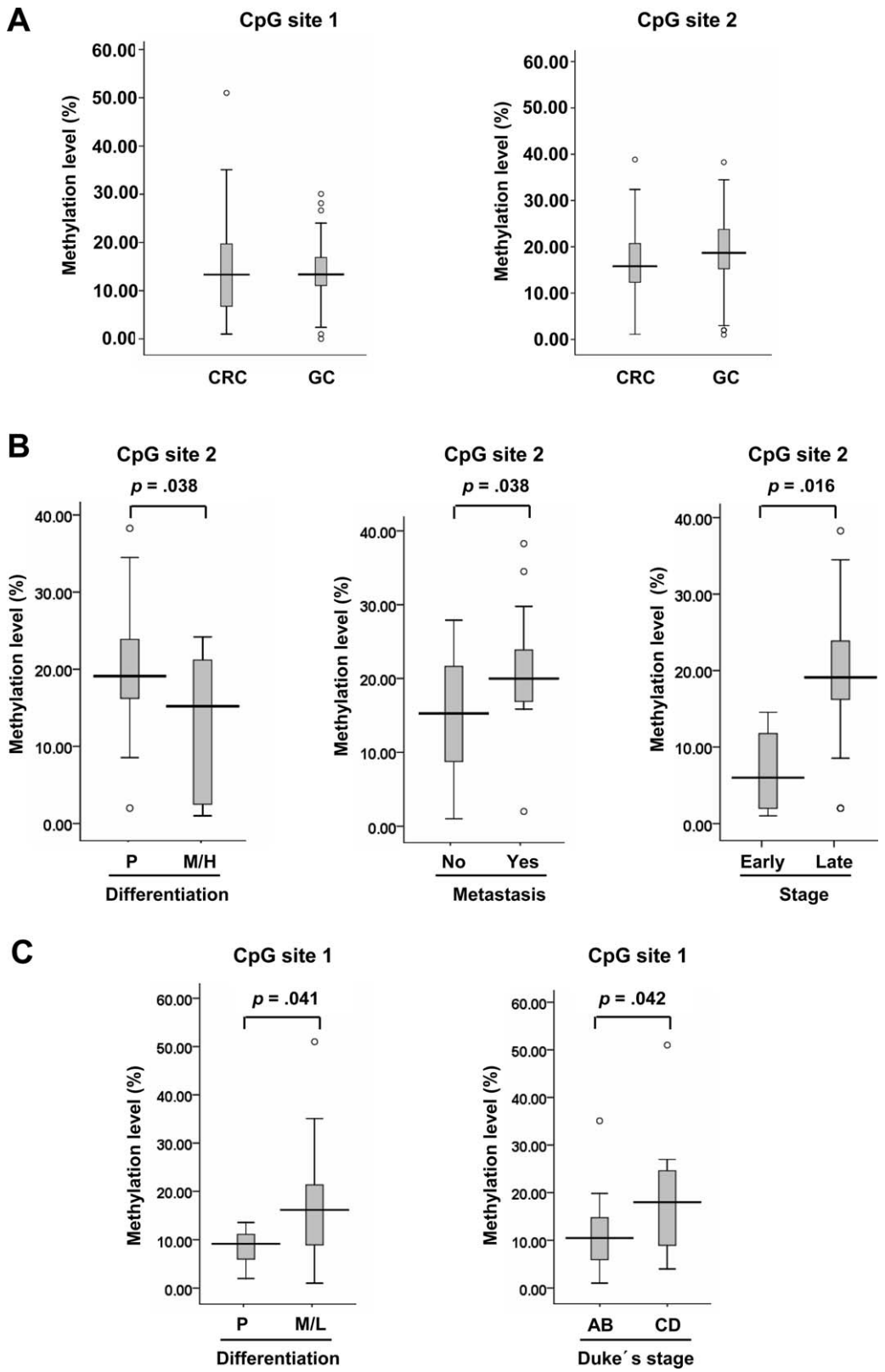


Figure 5. Comparisons of fecal *TERT* promoter methylation densities and clinical parameters of CRC and GC patients. **(A)**: Box plots showing *TERT* promoter methylation levels detected in CRC and GC patients at CpG sites 1 and 2. **(B)**: Comparison of *TERT* promoter methylation at CpG site 2 in GC patients with poor (P) or intermediate/high (M/H) differentiation; presence or absence of metastases and early or late stage. **(C)**: Comparison of *TERT* promoter methylation at CpG site 1 in CRCs with poor (P) or intermediate/high (M/H) differentiation as well as Duke's stage A/B or C/D.

Abbreviations: CRC, colorectal carcinoma; GC, gastric cancer.

relation to patient gender or age for CpG site 1 ($p = .369$ and $p = .204$, respectively), CpG site 2 ($p = .58$ and $p = .141$), and MetI ($p = .471$ and $p = .15$).

Increased methylation densities were observed in CRC and GC compared with healthy controls (Table 2). However, there was no difference in the methylation of CpG sites 1 and 2 between GC and CRC (Fig. 5A). In GC, the analyses revealed a significant association of higher methylated CpG site 2 (but not site 1) with poorly differentiated tumors, metastatic, and late stages of the disease (Table 1; Fig. 5B). When the MetI was used, significance remained with advanced and metastatic GC (Table 1). In CRC, higher levels of methylation at CpG site 1 (but not site 2) was significantly associated with poorly/intermediately differentiated tumors and advanced Duke's stages C and D (Table 1; Fig. 5C).

Discussion

Cancer-related *TERT* expression and telomerase activation is conceivably a specific biomarker for malignancies, and has been evaluated in both invasive and noninvasive assays. However, a number of issues impede reliable utility of the *TERT* mRNA or telomerase activity assay for diagnostic or screening purpose. First, infiltrated lymphocytes in tumors or exfoliated inflammatory cells have *TERT* expression, which may cause false-positive results [12, 23]. Second, telomerase and *TERT* mRNA are highly sensitive to temperature and inappropriate handling, and their quantification needs high-quality tissue samples, which limits their clinical application. Finally, specific *TERT* antibodies for immune-histochemical staining or immunoblotting are not available [24]. Given the above drawbacks, the need to develop alternative detection approaches is urgent. Recently, the *TERT* promoter mutation and aberrant methylation was identified in human malignancies but not in normal tissues/cells, which suggests that such mutation or methylation may be a cancer-specific marker [12, 23, 25–29]. Because DNA is sufficient for mutation and methylation analyses, its high stability makes the assay feasible. As the *TERT* promoter mutation rarely occurs in GIC [30], we sought to analyze whether the *TERT* promoter methylation profile differs between normal and malignant gastrointestinal tissues, and to evaluate whether the methylated *TERT* promoter is detectable in patient stool. We indeed identified the two methylation sites specific to CRC as reported by Choi [10]. Our pilot observation further showed that these methylated CpGs could serve as potential biomarkers in fecal DNA for GIC detection.

Aberrant *TERT* promoter methylation frequently occurs in epithelium-derived human malignancies such as breast, lung, prostate, and gastrointestinal cancer [28]. It should be pointed out here, that unlike other gene promoters where their methylation silences gene transcription or expression, the methylated *TERT* promoter is associated at certain sites with gene derepression and telomerase activation in malignant cells [10, 15, 19, 20]. We consistently found significantly higher levels of *TERT* promoter methylation in cancerous cells than in normal matched gastrointestinal tissues. Cancerous *TERT* mRNA levels were not measured in the present cohort of patients, although it is conceivable, based on previous studies by us and others, that the majority of them express *TERT* and acquire telomerase activation [9, 31–33].

Because early detection and timely management of patients with GIC is critical for better prognosis or survival, a screening program has long been recommended [3]. Fecal DNA analyses of methylated genes or gene promoters were previously tested for screening/diagnosis of GIC, and most of the examined targets were tumor suppressors; for example, *p16*, *SFRP2*, *RASSF1/2*, *TAPI2*, *GATA4/5*, and *NDRG4* [1, 3, 4, 34–36]. The methylated *TERT* promoter has so far not yet been evaluated as a fecal marker. Our proof-of-concept study suggests the feasibility of the *TERT* promoter methylation assay for GIC screening. The detection of those two methylated sites had sensitivity of approximately 50% at 90% specificity, comparable to other established fecal methylation markers [1, 4], while better than OB tests in GC. It is, however, evident from the present study that the *TERT* promoter methylation assay alone is insufficient. We observed that the combined detection of both fecal methylated TERT promoter and OB significantly increased AUC in CRC; however, the present results were obtained from a small cohort of patients and a further validation is apparently required. Nevertheless, a multitarget stool DNA test was recently shown to significantly increase sensitivity and specificity for CRC detection/screening [3]. Thus, the TERT promoter methylation may serve as a putative target in combination with other fecal biomarkers for CRC screening.

Higher levels of *TERT* expression and telomerase activity are usually associated with progressive malignancies or poor patient outcomes [37–39]. The present data showed that the presence of the highly methylated *TERT* promoter was closely associated with advanced or progressive GIC. It is thus worthy of testing whether the methylation status alone could serve as a prognostic factor in patient outcome prediction.

In addition to the methylation-based fecal detection for GIC screening, the assessment of GIC-specific DNA methylation biomarkers in blood have also been demonstrated to be promising for this screening purpose. For instance, plasma SEPT9 methylation assays were shown to detect the vast majority of CRC at all stages and colorectal locations with approximately 90% sensitivity and specificity [40, 41], which is superior to the fecal assay of the *TERT* promoter methylation. It is currently unclear whether the *TERT* promoter methylation in blood may serve as a useful biomarker for GIC screening, or whether the simultaneous detection of the *TERT* promoter methylation in both blood and feces improves the GIC screening efficiency, which calls for further investigations.

In China, GC is more prevalent than CRC and a leading cause of cancer-related death [42]; however, a GC screening program has not been established, which makes its early detection difficult. Therefore, effective early diagnosis for GC remains a significant challenge [42]. Our present finding may improve GC screening efficiency, but identification of more reliable biomarkers is apparently required to promote better detection of GC at an early stage, thereby resulting in favorable patient outcomes.

Conclusion

We show a highly significant difference in the *TERT* promoter methylation between normal and malignant gastrointestinal tissues, involving two specific methylated CpGs. Higher methylation densities at these two sites were significantly associated

with poor cellular differentiation and advanced stages or metastasis of GIC. Finally, this proof-of-concept study indicates that the methylated *TERT* promoter may be developed into a fecal biomarker for screening of GIC.

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Disclosures

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For Further Reading:

Donna Niedzwiecki, Rian M. Hasson, Heinz-Josef Lenz et al. A Study of Thymidylate Synthase Expression as a Biomarker for Resectable Colon Cancer: Alliance (Cancer and Leukemia Group B) 9581 and 89803. ***The Oncologist*** 2017;22:107-114; first published on November 7, 2016.

Implications for Practice:

This study finds that measurement of tumor levels of thymidylate synthase is not helpful in assigning specific adjuvant treatment for colorectal cancer. It also highlights the importance of using prospective analyses within treatment clinical trials as the optimal method of determining biomarker utility.